

## **REMARKS**

By this amendment, claims 1, 55-56 and 65-66 have been amended, and new claims 67-96 have been added. Claims 1 and 48-96 are under consideration.

### **Allowable subject matter**

Applicant thanks Examiner for the indication that claims 55 and 65 would be allowable if rewritten in independent form. Applicant has hereby amended claims 55 and 65 to include all the limitations of the base claims from which they depend, claims 1 and 57, respectively.

Further new claims 67-81 have been added to the application. New claims 67-74 depend from currently amended claim 55, and new claims 75-81 depend from currently amended claim 65. In both groups of new claims, embodiments of the types of cells which may be utilized in the practice of the present invention are recited: insect, plant, yeast, fungal, bacterial, and mammalian cells. Applicant submits that no new matter is introduced in these claims since page 13, paragraph #0163 of the application states that "Cells of interest include, but are not limited to, mammalian cells and non-mammalian cells, such as, for example, CHO, plant, yeast, bacterial, insect and the like." and original claim 45 of the application lists yeast, insect, fungal, plants and bacterial cells as types of cells that may be utilized in the invention.

Applicant respectfully submits that claims 55 and 65, as amended herein, and their dependent claims (67-75 and 76-83, respectively), are thus patentable. Applicant requests speedy review and allowance of these claims.

### **35 U.S.C 112 Rejections**

#### Second Paragraph

Claims 55 and 66 stand rejected under 35 U.S.C 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which application regards as the invention. Examiner states that the meaning of the word "human" is unclear. Claims 55 and 66 have hereby been amended to recite "...synthase gene isolated form a human source" instead of "human" as suggested by Examiner.

In view of the foregoing, Applicant respectfully request withdrawal of this rejection, and allowance of claims 55 and 66.

First Paragraph

Claims 1, 48-54, 56-64 and 66 stand rejected under 35 U.S.C.112, first paragraph, for lack of enablement. The Examiner states that the specification, while being “enabling for an isolated cell which is recombinant or genetically modified to contain or co-express a CMP-sialic acid synthase gene and a sialic acid phosphate synthase gene, where the cell is capable of producing donor substrate CMP-SA when supplemented with N-acetylmannosamine (ManNAc), above the levels produced before such genetic modification”, does not provide enablement for a cell without any supplementation with ManNAc. Consistent with Examiner’s comments, Claims 1 and 57 have hereby been amended to recite that the cell of claim is provided with ManNAc. New claims 84 and 89 recite that ManNAc is provided via addition of ManNAc to the media in which the cell is grown. Applicant submits that new claims 84 and 89 which recite the feature of supplementation with ManNAc as suggested by Examiner are thus patentable.

Applicant further submits that the specification of the present application also contains support for providing ManNAc via genetic engineering of the cell to have the ability to catalyze the synthesis of ManNAc. This embodiment of the invention is recited in new claims 85 and 90. In addition, this may be brought about by genetically engineering the cell to contain, in addition to the SAS and CMP-SA synthetase enzymes, an epimerase enzyme that catalyzes the production of ManNAc. Support for the genetic engineering method of increasing ManNAc levels in the cell may be found in the specification at, for example, page 16, paragraph 0170 which reads as follows: “According to the present invention, production of the donor substrate, CMP-SA, may be achieved by adding key precursors such as N-acetylmannosamine ManNAc ... to cell growth media [or] by *enhancing expression of limiting enzymes* in CMP-SA production pathway in the cells, or any combination thereof.” This method is supported in detail beginning with the last sentence of page 30 (“Alternatively, the enzymes...”) and continuing through paragraph 0218 on page 31. In this section of the specification, the concept of genetically engineering the cell of interest to contain a gene encoding an epimerase enzyme is discussed as option (a), “direct epimerization of GlcNAc”. The feature of genetically engineering the cells of the present invention to catalyze epimerization of ManNAc is recited in new claims 85 and 90. Applicants

submit that new claims 84-85 and 89-90 thus do not introduce any new matter.

In addition, Applicant herewith submits a manuscript authored by inventor Dr. Michael Betenbaugh in which experiments with cells that are genetically engineered to contain an epimerase are described. The experiments show that in cells which have been genetically engineered to contain both the SAS and epimerase enzymes, significantly elevated levels of Neu5Ac, the immediate precursor of CMA-SA, are produced.

Applicant notes that the cells described in the manuscript do not contain the final enzyme of the CMP-SA pathway, CMP-SA synthase, and CMP-SA was not measured. Rather, they contain the epimerase and SAS enzymes, and the product that was measured is the direct precursor of CMP-SA, i.e. Neu5Ac. Those of skill in the art will recognize however, that inclusion of the synthase gene in such cells is a routine matter and would allow conversion of the Neu5Ac to the final product, CMP-SA. This was shown in experiments described on page 93, paragraphs 0388-0390 of the application. New claims 86 and 91 recite genetically engineering the cell to contain an epimerase, and new claims 87-88 and 92-93 recite embodiments of the invention in which two different epimerases appropriate for use in the invention are recited (GlcNAc-2 and UDP-GlcNAc-2).

New claim 94 recites yet another feature of the invention. As stated on page 16 in paragraph of 0170 of the application, precursors of CMP-SA other than ManNAc may also be added to the growth medium, for example, GlcNAc. Further, as described in the last sentence of paragraph 0170 on page 16, several of the possible means of elevating precursor levels in the cells of the present invention may be employed in combination. New claim 94 recites the feature that the cells of the invention, which have also been genetically engineered to contain the SAS, CMP-SA synthase, and epimerase genes, also contain elevated levels of GlcNAc due to supplementation of the cell growth media with GlcNAc. The accompanying manuscript contains a description of cells which have been genetically transformed to contain both SAS and the epimerase genes, and for which the growth medium is supplemented with GlcNAc. As can be seen in the manuscript, these cells produce significantly elevated levels of Neu5Ac, the direct precursor of CMA-SA. As described in the preceding paragraph, the further inclusion of the

CMP-SA synthase gene in the cell by methods that are well established in the art would allow conversion of the Neu5Ac to CMP-SA.

In view of the foregoing, Applicant respectfully request withdrawal of this rejection, and allowance of claims 1 and 57 as amended, and new claims 84-94.

**New Claims 95 and 96.**

New claims 95 and 96 recite that embodiment of mammalian cells for claims 1 and 57, respectively. Applicant notes that other embodiments (yeast, insect, fungal, plants and bacterial) are given in claims 48-53 (dependent on claim 1) and claims 58-63 (dependent on claim 57). These dependent claims were added to the application in the previous amendment (filed April 28, 2003). Mammalian cells were inadvertently omitted from the dependent claims that were added at that time.

Support for new claims 95 and 96 is as set forth above in the section regarding Allowable Subject Matter, i.e. the concept of utilizing mammalian cells in the practice of the present invention is given on page 13, paragraph #0163 of the application, which reads “Cells of interest include, but are not limited to, mammalian cells...”.

Applicant respectfully requests consideration and allowance of new claims 95 and 96.

**Other amendments**

Claim 57 has hereby been amended to clarify the subject matter of the claim. Claim 57 previously recited that the isolated or purified cell of the claim was from a recombinant or genetically engineered cell line containing and co-expressing a CMP-SA synthase gene and an SAS gene to produce CMP-SA at a level that is higher than that of a corresponding “natural” cell line. Those of skill in the art will recognize that a more appropriate term for a “starting” cell line that is genetically engineered as described in the claim is the “parent” cell line. Thus, the term “natural” has been replaced by “parent”. The parent cell line may be any cell line that has not been genetically modified in the particular manner described in claim 57, for example a cell which has already been genetically engineered in a different manner, e.g., to contain enzymes other than CMP-SA synthase and SAS, to be resistant to antibiotics, to be fluorescent, etc. Those of skill in the art will recognize that a cell from such a parent cell line would serve as a proper reference for

the cell of claim 57. The same change has been incorporated into currently amended claim 65.

Claims 49 and 59 have been amended to correct a previous spelling error. The letter "h" was previously omitted in the first syllable of *Tricholpusia ni*.

**Formal Matters and Conclusion**

In view of the foregoing, Applicant submits that all rejections have been successfully traversed and that claims 1 and 48-91 should be deemed new and unobvious over the prior art of record. The Examiner is respectfully requested to reconsider and pass the above application to issue at the earliest possible time.

Should the Examiner find the application to be other than in condition for allowance, the Examiner is requested to contact the undersigned at the local telephone number listed below to discuss any other changes deemed necessary in a telephonic or personal interview.

Please charge any underpayment or credit any overpayment of fees to attorney's deposit account #50-2041.

Respectfully submitted,



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# Engineering the sialic acid synthesis pathway in insect cells: Identifying bottlenecks in the pathway and strategies to overcome them.



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**Keywords:** sialic acid 9-phosphate synthase, *Spodoptera frugiperda* (Sf9), Metabolic engineering, N-acetylneuraminic acid (Neu5Ac), KDN, UDP-GlcNAc epimerase/ ManNAc kinase

**Abbreviations:** The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; SAS, sialic acid 9-phosphate synthase; ManNAc, *N*-acetylmannosamine; Sf9, *Spodoptera frugiperda*; EpimKin, UDP-GlcNAc 2-epimerase/ ManNAc kinase; GlcNAc, *N*-acetylglucosamine; Ac<sub>4</sub>ManNAc, tetra-O-acetylated ManNAc; KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; BEVS, Baculovirus Expression Vector System; Man, Mannose; Gal, Galactose; CMP-Neu5Ac, cytidine monophosphate Neu5Ac; UDP-GlcNAc, uridine diphosphate GlcNAc; ManNAc-6-P, ManNAc-6-phosphate; Man-6-P, Mannose-6-phosphate; CMP-SAS, cytidine monophosphate sialic acid 9-phosphate synthase; CHO, Chinese hamster ovary; CMP-KDN, cytidine monophosphate 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; AcSAS, *Autographa californica* baculovirus containing sialic acid-9-phosphate synthase gene; DMB, 1,2-diamino-4,5-methylene dioxybenzene dihydrochloride; HPLC, high performance liquid chromatography; PEP, phosphoenolpyruvate; Neu5Ac-9-P, *N*-acetylneuraminic acid 9-phosphate; AcEpimKin, *Autographa californica* baculovirus containing UDP-GlcNAc 2-epimerase/ ManNAc kinase gene; AcKin, *Autographa californica* baculovirus containing mutated UDP-GlcNAc 2-epimerase/ ManNAc kinase gene with only ManNAc kinase activity; AcEpim, *Autographa californica* baculovirus containing mutated UDP-GlcNAc 2-epimerase/ ManNAc kinase gene with only 2-epimerase activity.

## ABSTRACT

Previous studies have indicated negligible intracellular levels of both sialylation and precursor *N*-acetylneurameric acid (Neu5Ac) in a number of insect cell lines grown in serum-free medium. The overexpression of the human sialic acid-9-phosphate synthase (SAS) in combination with *N*-acetylmannosamine (ManNAc) feeding has been shown to overcome this limitation. In this study we evaluated the potential bottlenecks in the sialic acid synthesis pathway in a *Spodoptera frugiperda* (Sf9) insect cell line and devised strategies to overcome them by overexpression of the enzymatic pathway enzymes combined with appropriate substrate feeding. Co-expression of SAS and UDP-GlcNAc 2-epimerase/ ManNAc kinase (EpimKin), the bifunctional enzyme initiating sialic acid biosynthesis in mammals, resulted in Neu5Ac synthesis without use of any external media supplementation to demonstrate that Neu5Ac could be generated intracellularly in Sf9 cells using natural metabolic precursors. *N*-acetylglucosamine (GlcNAc) feeding in combination with this co-expression resulted in much higher levels of Neu5Ac compared to levels obtained with ManNAc feeding with SAS expression alone. The lower Neu5Ac levels with ManNAc feeding suggested limitations in the transport and phosphorylation of ManNAc. The bottleneck in phosphorylation was likely due to utilization of GlcNAc kinase for phosphorylation of ManNAc in insect cells and was overcome by expression of ManNAc kinase. The transport limitation was addressed by addition of tetra-*O*-acetylated ManNAc ( $\text{Ac}_4\text{ManNAc}$ ), which is easily taken up by the cells. An alternative sialic acid, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN), could also be generated in insect cells suggesting the potential for controlling not only the production of sialic acids, but also the type of sialic acid generated. The levels of KDN could be increased with virtually no Neu5Ac generation when Sf9 cells were fed excess GlcNAc. The results of these studies may be used to enhance the sialylation of target glycoproteins in insect and other eukaryotic expression systems.

## Introduction

Insect cells are widely used for the production of numerous recombinant proteins, typically through the Baculovirus Expression Vector System (BEVS). They often produce recombinant proteins in high yields and have the capacity to perform post-translational modifications including glycosylation (1). The nature of the attached glycan can influence the structure, function and stability of the protein (2, 3). Unlike mammalian cells, insect cells are typically incapable of generating complex *N*-glycans with a terminal *N*-acetylneuraminc acid (Neu5Ac) (4, 5). Insect cells often produce truncated or paucimannosidic *N*-glycans terminating in mannose (Man) or occasionally *N*-acetylglucosamine (GlcNAc) (4, 6). As a result, glycoproteins derived from insect cells are likely to have shorter *in vivo* circulatory half-lives which lower the potential therapeutic value of these products (7). An insect cell line capable of producing sialylated proteins while retaining the other advantages of the BEVS such as high yields would represent a significant development to the biotechnology industry.

Metabolic engineering represents one approach for improving the glycosylation properties of insect cell proteins. Previous efforts in this area have primarily focused on expression of transferases. *N*-acetylglucosaminyltransferase-I (GlcNAcT-I) (8) and  $\beta$ -1, 4-galactosyltransferase (GalT) (9, 10) have been expressed in insect cells to improve the levels of GlcNAc and Galactose (Gal) appearing on insect cell glycoproteins. Recently, the combined expression of *N*-acetylglucosaminyltransferase-II (GlcNAcT-II) and GalT in a *Trichoplusia ni* (TN-5B1-4) insect cell line resulted in glycoproteins containing more than 50% of structures that were fully galactosylated on both branches (11). This cell line generated only paucimannosidic structures terminating in Mannose (Man) with a few structures containing GlcNAc on one of two Man branches in the absence of these heterologous transferases (4). Sialyltransferases have also been expressed in combination with galactosyltransferases in both mammalian and insect cells to improve sialylation of the proteins (12, 13). However, the expression of these transferases will be most effective if there is an adequate supply of the substrates, the activated sugar-nucleotides, which are utilized in the galactosylation and sialylation reactions.

The primary donor substrate for sialylation is CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) and the metabolic pathway used by mammalian cells for generating CMP-Neu5Ac and its dedicated metabolic precursor, Neu5Ac, is shown in Fig. 1A. UDP-*N*-acetylglucosamine (UDP-GlcNAc) generated from basic metabolites is first converted to ManNAc by the 2-epimerase activity of the bifunctional enzyme UDP-GlcNAc-2-epimerase/ ManNAc kinase (EpimKin). ManNAc is then phosphorylated to ManNAc-6-phosphate (ManNAc-6-P) by the kinase activity present on the same enzyme (14). ManNAc-6-P is the substrate for sialic acid-9-phosphate synthase (SAS) to synthesize Neu5Ac-9-phosphate (15). The same enzymes can also yield alternate sialic acid, KDN-9-phosphate using Man-6-phosphate (Man-6-P) as substrate (Fig. 1B). Unknown specific or non-specific dephosphorylases act on these intermediates to yield the Neu5Ac and KDN, which are in turn are converted to CMP-Neu5Ac and CMP-KDN by CMP-sialic acid synthase (CMP-SAS) (16). These CMP-sialic acids are the activated molecules that transfer the sialic acid to the glycan on the proteins. As an alternative, *N*-acetylmannosamine (ManNAc), the first dedicated precursor of Neu5Ac has been added to CHO cells culture media to enhance sialylation of interferon- $\gamma$  (17). This same pathway for sialic acid synthesis has been shown to utilize other unnatural ManNAc derivatives and synthesize unnatural analogs of sialic acid (18). The promiscuous nature of the pathway has been exploited to introduce reactive functional groups such as ketones (19) and azides (20) on the modified sialic acids at the cell surface. In addition, sialic acid when supplemented to certain eukaryotic cell lines media has been shown to be incorporated into glycoproteins (21). Sialylated structures have been detected in insect cells expressing recombinant galactosyltransferases and sialyltransferases (22, 23). However, supplementation of the culture medium with serum, which is rich in sialic acids (24), was necessary to obtain these sialylated forms.

Applying metabolic engineering strategies to include the enzymes involved in sialic acid metabolism and transport represents another approach for altering the levels of necessary substrates for sialylation. Previous studies in insect cells have indicated negligible intracellular levels of endogenous sialic acids including Neu5Ac (15) and CMP-sialic acids, the active substrate for sialylation (25, 26). The overexpression of the human SAS in combination with supplementation of ManNAc has been shown to

overcome the absence of detectable Neu5Ac production in these insect cells (15). Interestingly, the insect cells apparently included endogenous specific or non-specific kinase activities to phosphorylate ManNAc and dephosphorylate the NeuAc-9-phosphate product of the synthase reaction shown in Fig. 1A. Similarly, the expression of both human SAS and CMP-SAS in combination with ManNAc feeding was sufficient to generate intracellular CMP-sialic acids in insect cells (16). In addition to Neu5Ac, the insect cells generated the alternative sialic acid, 2-Keto-3-deoxy-D-glycero- D-galacto -nononic acid (KDN) (15). Following expression of SAS, KDN-9-phosphate was synthesized by conversion of endogenous Man-6-P in the presence or absence of fed ManNAc. This KDN-9-phosphate is then converted to KDN by endogenous phosphatases present in insect cells. Similarly, CMP-KDN was formed by co-expression of CMP-SAS and SAS (16) (Fig. 1B).

In the present study we further examined the metabolic pathways for sialic acid production in insect cells. Using the mammalian sialic acid synthesis pathway as a framework we have identified the bottlenecks that exist in the sialic acid synthesis in insect cells. To elucidate bottlenecks, we incorporated the genes for selective enzymes of the pathway in combination with appropriate substrates. *Spodoptera frugiperda* (Sf9) cells were infected with baculoviruses containing the sialic acid 9-phosphate synthase gene (AcSAS) in combination with the AcEpim (baculovirus containing functional UDP-GlcNAc 2- epimerase gene without ManNAc kinase activity), AcKin (baculovirus containing functional ManNAc kinase gene without UDP-GlcNAc 2-epimerase activity), and AcEpimKin (baculovirus containing UDP-GlcNAc 2-epimerase/ ManNAc kinase bifunctional gene) in the presence or absence of ManNAc and GlcNAc in the media. By suitable infections and substrate feeding, we were able to overcome specific pathway limitations and enhance sialic acid production as well as control the ratio of the two different sialic acids (Neu5Ac and KDN) synthesized by the cells.

## Materials and Methods

*Preparation of sugar solutions.* Sugar solutions of ManNAc, GlcNAc (0.1 M and 1 M each) (Sigma chemicals, St. Louis, MO) were prepared by dissolving them in water followed by filter sterilization.

Tetra-*O*-acetylated ManNAc (Ac<sub>4</sub>ManNAc) was prepared by following a protocol adapted from a previously published procedure (27). Briefly, 7.0 mmol of ManNAc (Pfanstiel, Waukegan, IL) was dissolved in 30 ml of a 2:1 solution of pyridine and acetic anhydride. The mixture was stirred at room temperature for 12 hrs. Subsequently the solvent was removed *in vacuo*, and the residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and was washed successively with 30 ml of concentrated HCl (twice), and then 30 ml of saturated Na<sub>2</sub>SO<sub>4</sub>. Finally the mixture was dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The residue was purified by silica gel chromatography, eluting with a gradient of 2:1 to 1:2 hexanes/EtOAc. The resulting white amorphous foam was characterized by NMR as a mixture of anomers. The product was stored at -20 °C; periodically 100 mM stock solutions of tetra-*O*-acetylated ManNAc were prepared in ethanol and stored at 4 °C until use.

*Cell culture.* Sf9 (ATCC, Manassas, VA) cells were grown in serum-free HyClone SFX media in shaker flasks. Approximately 0.8 × 10<sup>6</sup> Sf9 cells taken from cell cultures at densities between 1.5-2.5 × 10<sup>6</sup> cells/ml were plated on each well of a 6-well plate. After attachment, the medium was removed and 2 ml of fresh medium was supplied to each well. Cells were infected with 20 µl of each virus or left uninfected. Baculovirus infections were performed as described in O'Reilly *et al.*, (1992) (1).

The medium was supplemented by the appropriate sugar solution at the time of infection. For ManNAc supplementation, Sf9 cells were grown in media supplemented with 0.1, 2, 5 mM ManNAc (from the 0.1 M ManNAc stock) and 10, 20, 50 mM ManNAc (from the 1 M ManNAc stock). For GlcNAc supplementation, Sf9 cells were grown in media supplemented with 0.1, 2, 5 mM GlcNAc (from 0.1 M GlcNAc stock) and 10, 20 mM GlcNAc from the (1 M GlcNAc stock). For Ac<sub>4</sub>ManNAc supplementation, the cells were grown in medium supplemented with 0.1, 0.2, 0.5, 1 mM Ac<sub>4</sub>ManNAc (from 100 mM Ac<sub>4</sub>ManNAc).

Cells were harvested 72 hrs post-infection by removing cell culture medium and washing cells twice with phosphate buffered saline (Life Technologies, Bethesda, MD). Cells were vortexed then sonicated with a Tekmar sonic disruptor (Cincinnati, OH) for 30 s at 50% cycle at a power setting of 2.5. Samples

were analyzed for total protein content with a Pierce BCA assay kit (Rockford, IL) and a 96-well plate reader (Molecular Devices, Sunnyvale, CA), and analyzed for sialic acids as described below.

*Neu5Ac/KDN Detection.* Previous studies have indicated the absence of sialic acid on cellular proteins for Sf-9 cells lacking the expression of both heterologous galactosyltransferase and sialyltransferase genes (13, 23). Consequently, any incorporation of Neu5Ac or KDN into cell proteins was ignored. The free sialic acid content was measured by the procedure as described in Hara *et al.*, (1989) (24). 95 µl of 1, 2-diamino-4, 5-methylene dioxybenzene dihydrochloride (DMB; Sigma) reducing solution (7.0 mM DMB in 1.4 M acetic acid, 0.75 M  $\beta$ -mercaptoethanol, and 18 mM sodium hydrosulfite) was added to 5 µl of sample and incubated at 50 °C for 2.5 hrs from which 3-10 µl was used for HPLC analysis on a Shimadzu (Columbia, MD) VP series HPLC using a Waters (Milford, MA) Spherisorb 5-µm ODS2 column. A Shimadzu RF-10AXL fluorescence detector with 448-nm emission and 373-nm excitation wavelengths was used for detecting peaks. An acetonitrile, methanol, and water mixture (9:7:84, v/v/v) with a flow rate of 0.7 ml/min was the mobile phase. Response factors of Neu5Ac and KDN were established with authentic standards based on peak areas for quantifying sample sialic acid levels. Sialic acid content was normalized based on the total protein content measured with the Pierce BCA assay kit and a Molecular Devices (Sunnyvale, CA) microplate reader.

*GlcNAc kinase/ ManNAc kinase activity measurement.* Enzyme activities were determined as described earlier (28). Briefly, assays contain 60 mM Tris/HCl, pH 7.5, 20 mM MgCl<sub>2</sub>, 5 mM GlcNAc/ManNAc, 50 nCi <sup>14</sup>C-GlcNAc/<sup>14</sup>C-ManNAc, 20 mM ATP in a final volume of 200 µl. Assays were run 30 min at 37°C and stopped by addition of 300 µl ethanol. Radiolabeled substrates were separated by descendent paper chromatography, and radioactivity was determined in a Tri-Carb 1900A liquid scintillation counter (Packard).

## Results

### *Effect of ManNAc media supplementation on the sialic acid levels in AcSAS infected cells*

ManNAc is a metabolic precursor in the biosynthesis of Neu5Ac as shown in Figure 1A. This conversion involves the phosphorylation of ManNAc to ManNAc-6-P, which then fuses with phosphoenolpyruvate (PEP) to generate Neu5Ac-9-P. This intermediate is subsequently dephosphorylated to give Neu5Ac. In previous studies, Sf9 cells grown in serum free media were observed to include negligible levels of Neu5Ac (15). In order to determine if insect cells could be engineered to produce Neu5Ac using N-acetylmannosamine (ManNAc) substrates, Sf9 cells were infected with the AcSAS baculovirus containing the sialic acid-9-phosphate synthase (sas) gene and supplemented with ManNAc at the time of infection. As shown in Figure 2A, these AcSAS-infected cells generated significant levels of Neu5Ac that varied with the amount of fed ManNAc. In the absence of ManNAc supplementation, however, the levels of Neu5Ac were very low (approximately 100 fmol/ $\mu$ g total protein), suggesting that Sf9 cells have a limitation in the amount of ManNAc available for Neu5Ac synthesis. As ManNAc supplementation levels were increased, the levels of Neu5Ac synthesized increased over the entire range up to 27000 fmol/ $\mu$ g total protein with 50 mM ManNAc supplementation as shown in Figure 2A. However, the increase in Neu5Ac obtained began to slow around 20 mM of fed ManNAc, and there was only a relatively small increase in the Neu5Ac levels in changing from 20 mM to 50 mM ManNAc supplementation to the media.

A similar experiment was done with uninfected Sf9 cells and Sf9 cells infected with a control virus without any foreign gene (A35). The Neu5Ac levels in the uninfected cells as well as the cells infected with the A35 (negative control) virus also increased with ManNAc feeding (Fig. 2B), with the same dependency on sugar concentration. However, these quantities were more than two orders of magnitude below those seen with the AcSAS infection. This difference suggests a native, albeit low, sialic acid 9-phosphate synthase activity in Sf9 cells. The lowest levels of Neu5Ac were observed following infection of the A35 virus and may reflect the negative effects of viral infection on host cell functions.

In addition to Neu5Ac, the levels of the alternate sialic acid, KDN were also measured for these ManNAc feeding experiments. The sialic acid, KDN, is synthesized by SAS with Man-6-P and PEP as

substrates. In A35 infected and uninfected (*data not shown*) cells, KDN levels were not detected (Fig. 2C). In Sf9 cells infected with AcSAS, the KDN levels were detectable at levels between 170 and 260 fmol/  $\mu$ g total protein (Fig. 2C). The levels of intracellular KDN decreased slightly as ManNAc was fed but still remained above 150 fmol/  $\mu$ g protein. When the production of Neu5Ac relative to KDN is calculated, the Neu5Ac to KDN ratio rises rapidly with the initial addition of ManNAc due in large part to the rapid increase in Neu5Ac production accompanying ManNAc feeding (Fig. 2C).

*Effect of co-infecting UDP-GlcNAc 2-epimerase/ ManNAc kinase virus (AcEpimKin) and sialic acid 9-phosphate synthase virus (AcSAS)*

An alternative to ManNAc supplementation is to engineer the cells with the capacity to generate ManNAc, since Sf9 cells have negligible endogenous capacity to synthesize ManNAc (15). In mammalian cells ManNAc is synthesized *in vivo* by the epimerization of UDP-GlcNAc using UDP-GlcNAc 2-epimerase. The ManNAc is then converted to ManNAc-6-P by a kinase encoded on the same bifunctional complex (Fig. 1A). The bifunctional rat enzyme (Fig. 3) that generates ManNAc-6-phosphate from UDP-GlcNAc has been cloned into baculovirus (AcEpimKin), and infection of Sf9 cells with AcEpimKin results in the production of the active enzyme (29). To determine if Sf9 cells could be engineered to produce Neu5Ac without substrate supplementation, Sf9 cells were co-infected with the AcEpimKin and AcSAS baculoviruses simultaneously and then analyzed for intracellular sialic acid. Cells infected with AcEpimKin alone had negligible levels of Neu5Ac (Fig. 4) as did cells infected with AcSAS alone (Fig. 2A). However, co-infection with AcSAS and AcEpimKin enabled the cells to generate significant (16,900 fmol/  $\mu$ g) Neu5Ac intracellularly. This co-infection indicates that Sf9 cells generate the essential UDP-GlcNAc precursor for the synthesis of ManNAc and Neu5Ac endogenously. Thus cells infected with AcEpimKin and AcSAS can synthesize Neu5Ac in the absence of any additional media supplementation. In fact, the Neu5Ac levels observed in the co-infected cells were comparable to the levels observed in the AcSAS-infected cells supplemented with 10 mM ManNAc (Fig. 4).

### *Effects of GlcNAc feeding on Neu5Ac production*

GlcNAc feeding represents another possible alternative for increasing the synthesis of sialic acid in insect cells. The substrate for AcEpimKin is UDP-GlcNAc, and a 10-fold increase in the UDP-GlcNAc levels was found with 10 mM GlcNAc supplementation to the media (*data not shown*). To see the effect of GlcNAc feeding on sialic acid production, cells were co-infected with AcEpimKin and AcSAS in the presence of different concentrations of GlcNAc (Fig. 5). The amount of Neu5Ac generated increased with higher concentrations of GlcNAc reaching a level of 115,000 fmol/ µg of protein at 10 mM fed GlcNAc. This Neu5Ac level was more than six times higher than the amount of Neu5Ac generated when cells were infected with AcSAS and fed 10mM ManNAc.

### *Effects of infection with AcKin, a virus with only ManNAc kinase activity:*

Insect cells are capable of phosphorylating ManNAc to give ManNAc-6-P as evidenced by the generation of Neu5Ac in cells infected with AcSAS and supplemented with ManNAc. The sialic acid phosphate synthase (SAS) enzyme acts almost exclusively on ManNAc-6-P substrates (15). Previous studies showed that mammalian cells lacking the specific ManNAc kinase still display ManNAc kinase activity, which is derived from a secondary activity of GlcNAc kinase (30). To investigate if such an activity exists in insect cells as well, the ManNAc kinase and GlcNAc kinase activities in Sf9 cells were determined. GlcNAc kinase activity was found to be 4.6 mU/mg and ManNAc kinase activity 2.9 mU/mg. In the presence of equimolar ratios of GlcNAc and ManNAc, the ManNAc kinase activity dropped to less than 10% of its value measured in the absence of GlcNAc. This behavior is consistent with the activity observed for a mammalian GlcNAc kinase (30) and confirms that the ManNAc kinase activity in Sf9 cells is likely to be derived from the endogenous insect GlcNAc kinase.

The high levels of Neu5Ac generated when ManNAc-6-phosphate is synthesized intracellularly using UDP-GlcNAc 2-epimerase/ ManNAc kinase suggests a possible limitation in the production of ManNAc-6-P from the ManNAc added to the cell culture media. Even when ManNAc is fed up to 50

mM, the level of Neu5Ac saturates at 25,000 fmol/ µg protein as compared to 115,000 fmol/ µg found for co-infection with AcSAS and AcEpimKin in the presence of 10 mM GlcNAc feeding. The limitation could be in the intracellular enzymatic synthesis of ManNAc-6-phosphate from ManNAc, in the uptake of ManNAc by the cells, or both. To examine for a limitation in the ManNAc kinase activity which is responsible for the synthesis of ManNAc-6-P from ManNAc, the cells were infected with an AcKin virus along with AcSAS in the presence of variable amounts of fed ManNAc. AcKin is a baculovirus that expresses a UDP-GlcNAc-2-epimerase/ ManNAc kinase gene that contains unchanged ManNAc kinase activity but lacks any 2-epimerase activity due to the H45A point mutation in the epimerase domain of the bifunctional enzyme (Fig. 3) (29). The Neu5Ac production levels for co-infections with AcSAS and AcKin were then compared with cells infected AcSAS alone in the absence of any recombinant ManNAc kinase activity (Fig. 6). At levels of ManNAc supplementation 10 mM and below, the Neu5Ac yields were similar in the presence or absence of the additional AcKin infection, suggesting, that the ManNAc kinase activity of Sf9 GlcNAc kinase is sufficient for ManNAc phosphorylation. However, at levels of ManNAc 20 mM and above, the Neu5Ac levels were significantly higher in the AcKin co-infected cells, reaching a level of Neu5Ac that was more than 2.5 fold higher at 50 mM ManNAc. This increase in Neu5Ac production in the presence of recombinant ManNAc kinase suggests a limitation in the endogenous ManNAc kinase activity of Sf9 cells at higher ManNAc feeding levels. However, even though the Neu5Ac levels increased with the AcKin infection and ManNAc supplementation, the total Neu5Ac yields were not as high as those obtained with AcEpimKin and AcSAS co-infection in the presence of fed GlcNAc, indicating other possible limitations in the conversion of fed ManNAc to Neu5Ac.

#### *Effect of feeding tetra-O-acetylated ManNAc (Ac<sub>4</sub>ManNAc) on Neu5Ac production*

In order to examine for a possible transport limitation in the uptake of ManNAc by insect cells, the ManNAc was tetra-O-acetylated prior to being provided as a substrate to the medium. O-acetylation of ManNAc is known to facilitate its uptake in mammalian cells (31). O-acetylation involves the chemical

modification of the hydroxyl groups to acetyl groups (Fig. 7A). This chemical modification causes the ManNAc to be more lipophilic, which in turn allows the resulting Ac<sub>4</sub>ManNAc to diffuse more easily through the cell membrane. Once inside the cell the Ac<sub>4</sub>ManNAc is deacetylated to free ManNAc by non-specific esterases in the cytosol and endoplasmic reticulum (31).

In order to investigate for a possible limitation in ManNAc transport, Sf9 cells cultured in serum-free media were supplemented with 0.2 mM Ac<sub>4</sub>ManNAc and then co-infected with AcSAS and AcKin. The Neu5Ac level obtained using Ac<sub>4</sub>ManNAc was comparable to the amount from cells co-infected with AcEpimKin and AcSAS in presence of an equivalent level (0.2mM) of GlcNAc. Furthermore, this value was slightly higher than the amount of Neu5Ac obtained with same viruses (AcSAS and AcKin) supplemented with 10 mM ManNAc (Fig. 7B). The production of slightly higher levels of Neu5Ac using much lower levels of fed Ac<sub>4</sub>ManNAc compared with free ManNAc suggests a limitation does exist in the ManNAc transport into insect cells, and this transport limitation inhibits the cells ability to generate high levels of intracellular Neu5Ac. Unfortunately, Ac<sub>4</sub>ManNAc is also lethal to insect cells at elevated levels. For example, 1 mM Ac<sub>4</sub>ManNAc supplementation resulted in the death of majority of the cells after 2-3 days. Consequently, addition of Ac<sub>4</sub>ManNAc was limited to experiments in which the Ac<sub>4</sub>ManNAc concentration was held at or below 0.2 mM.

#### *KDN production in insect cells*

Neu5Ac is the predominant form of sialic acid generated when the AcSAS infection is carried out in the presence of ManNAc or following co-infection with AcEpimKin. The alternate sialic acid, KDN, is also synthesized albeit at much lower levels, by this same SAS enzyme using Man-6-P as the substrate (15) (Fig. 1B). Man-6-P is generated by phosphorylation of Man in cells. In order to determine if the levels of KDN could be increased by substrate addition, additional Man was added to the medium. However, feeding Man did not alter the KDN levels significantly (*data not shown*). This absence of any effect on KDN production with Man feeding could be due to high natural intracellular Man-6-P levels naturally or because high levels of Man are present in serum-free media used. As an alternative,

methods to increase KDN production relative to Neu5Ac were evaluated through the inhibition of the synthesis of ManNAc-6-P. As shown in Fig. 1A GlcNAc is incorporated into the metabolic pathway as GlcNAc-6-phosphate. The phosphorylation of GlcNAc was carried out in Sf9 by GlcNAc kinase, which also includes a secondary ManNAc kinase activity. Previously, we observed that feeding an excess of GlcNAc could restrict the ManNAc kinase activity of GlcNAc kinase and limit the levels of ManNAc-6-P available to the cells. Therefore GlcNAc feeding was considered as a possible method to limit Neu5Ac production in insect cells. Sf9 cells were co-infected with AcSAS and AcEpim (with and without AcKin) in the presence of fed GlcNAc and the production of Neu5Ac and KDN was measured. AcEpim is a baculovirus that expresses a UDP-GlcNAc-2-epimerase/ManNAc kinase that contains unchanged epimerase activity but lacks any ManNAc kinase activity due to the R420M point mutation in the kinase domain of the gene for this bifunctional enzyme (29). Also this mutant enzyme is feedback inhibited completely by 0.1 M CMP-Neu5Ac (29). In cells infected with AcKin along with AcEpim and AcSAS the cells synthesized high levels of Neu5Ac with GlcNAc feeding as expected (Fig. 8). In contrast, in GlcNAc-fed cells infected with AcEpim and AcSAS in the absence of AcKin infection, the synthesis of Neu5Ac was limited to negligible levels and KDN became the predominant sialic acid synthesized (Fig. 8).

## Discussion

Previous research in our laboratories has demonstrated that insect cells can be engineered to produce sialylation substrates (15, 16). The present work focused on identifying particular bottlenecks that may exist in the sialic acid synthesis pathways of insect cells. In particular, we focused on the production of the sialic acids Neu5Ac and KDN in an engineered Sf9 insect cell line. By varying the specific pathway genes as well as the substrates involved, we determined that particular processing steps can indeed limit the production of sialic acid. Furthermore, a suitable combination of substrate feeding alternatives and expression of various genes can be used to control the levels of sialic acid as well as the type of sialic acid formed.

As previously reported by Lawrence et al. (2000) (15), Sf9 cells synthesize Neu5Ac and KDN when infected with a baculovirus carrying the gene for sialic acid 9-phosphate synthase in the presence of exogenously fed ManNAc. In this study, the levels of Neu5Ac were observed to increase with ManNAc supplementation up to 20 mM fed ManNAc. This increase in Neu5Ac production clearly indicates a limitation in the available ManNAc for Neu5Ac synthesis in Sf9 cells. However, the addition of 50 mM ManNAc gave only a 12 % increase in the synthesis of Neu5Ac over the level obtained with 20 mM ManNAc. Thus, a bottleneck in the sialic acid pathway is present in insect cells such that increasing the level of ManNAc present in the medium above 20 mM does not cause a significant enhancement in the amount of Neu5Ac generated. This bottleneck could exist either at the step involving ManNAc transport into the cells or in the metabolic conversion of ManNAc to substrates which can be utilized by the sialic acid synthesis enzyme. Nonetheless, the intracellular Neu5Ac content was still over 100 times higher in the AcSAS-infected lysates as compared to control culture lysates. Even so, the presence of detectable Neu5Ac in control cultures suggests that insect cells may contain very low endogenous levels of the enzymes for sialic acid synthesis. The gene for sialic acid synthesis has indeed been detected in *Drosophila melanogaster* (32) although the endogenous enzymatic activity was undetectable in Schneider S2 cell lines. KDN, an alternate sialic acid, was also generated in the current studies following AcSAS infection. The ratio of KDN to Neu5Ac decreased drastically following ManNAc feeding due to a rapid increase in the synthesis of Neu5Ac, indicating that ManNAc 6-P is the preferred substrate of SAS.

To determine if ManNAc feeding could be avoided and if insect cells could be engineered to generate Neu5Ac completely from intracellular metabolites, insect cells were co-infected with the AcEpimKin and AcSAS virus without any media supplementation. The co-infections of AcEpimKin and AcSAS were performed with the intent of avoiding potentially costly ManNAc feeding by introducing the enzymatic machinery for ManNAc production into the cells. The bifunctional enzyme UDP-GlcNAc 2-epimerase/ ManNAc kinase (29) present on AcEpimKin produces ManNAc and then ManNAc-6-P, the precursor for the SAS enzyme, from UDP-GlcNAc. Previous studies have indicated that Sf9 cells have

significant levels of the UDP-GlcNAc present intracellularly (26). Indeed, the generation of Neu5Ac in the presence of AcSAS and AcEpimKin indicates that metabolic engineering can be used to complete the sialic acid synthesis pathway and that sialic acid can be synthesized in cells without any additional substrates. In fact, co-infection of AcSAS and AcEpimKin resulted in the production of Neu5Ac levels comparable to those seen with AcSAS infection and 10 mM ManNAc feeding. Furthermore, the co-infection of AcSAS and AcEpimKin in cells supplemented with 10 mM GlcNAc (a more cost-effective reagent than ManNAc) generated levels of Neu5Ac that were around six times higher than those from the co-infection of AcSAS and AcEpimKin without additional substrates and in fact saturated the experimental Neu5Ac detection system. These high levels of Neu5Ac observed with GlcNAc feeding also indicate that GlcNAc feeding is much more efficient for Neu5Ac generation as compared to ManNAc feeding.

The AcEpimKin and AcSAS co-infection results present further evidence that a bottleneck to Neu5Ac synthesis exists following ManNAc feeding. The presence of efficient synthesis of high levels of Neu5Ac from intracellularly generated ManNAc-6-P in these studies suggests that the bottleneck to Neu5Ac production for exogenously supplied ManNAc occurs either in the ability of insect cells to uptake or phosphorylate ManNAc. Although GlcNAc feeding led to higher levels of Neu5Ac than ManNAc feeding, understanding the bottleneck in the Neu5Ac synthesis from fed ManNAc is useful due to the feedback inhibition of the epimerization of UDP-GlcNAc by CMP-Neu5Ac (33). As a result, generation of high amounts of CMP-Neu5Ac may require the epimerization step to be bypassed and thus may ultimately depend on ManNAc feeding. In order to identify the bottleneck in the synthesis of Neu5Ac with fed ManNAc, both ManNAc transport from the media to the cells and the phosphorylation of ManNAc by ManNAc kinase were examined.

In the absence of any recombinant ManNAc kinase activity, Neu5Ac was still synthesized in cells infected with AcSAS and supplemented with ManNAc to indicate that the ManNAc was converted to ManNAc-6-P. As suggested previously in mammalian cells, the ManNAc kinase activity in insect cells was found to be derived from GlcNAc kinase (30). Indeed, when the ManNAc kinase activity was

measured in the presence of equimolar ratios of GlcNAc and ManNAc, the ManNAc kinase activity fell to 10% of the activity found in the absence of GlcNAc. However, even though GlcNAc kinase can perform this enzymatic step, GlcNAc kinase is likely to be much less efficient than the activity obtained using a specific ManNAc kinase. We found that infection of the cells with AcKin (baculovirus containing the ManNAc kinase gene) in combination with AcSAS resulted in higher yields of Neu5Ac, especially at ManNAc levels greater than 20 mM. With 50 mM ManNAc feeding the co-infection of AcKin and AcSAS resulted in Neu5Ac levels more than 2.5 times higher levels than those found with AcSAS infection alone. This increase in Neu5Ac indicated that, in the absence of AcKin, a bottleneck exists in the phosphorylation of exogenously supplied ManNAc especially at higher concentrations of fed ManNAc.

Despite overcoming the limitation in ManNAc kinase activity using AcKin and AcSAS, the levels of Neu5Ac in insect cells were still found to be lower than those with infection with the AcEpimKin and AcSAS viruses complemented with GlcNAc feeding. To determine if this limitation was due to poor uptake of ManNAc by the cells, the supplemented ManNAc was modified by *O*-acetylation in an effort to enhance the efficiency of transport. In Jurkat cells *O*-acetylated compounds have been shown to be utilized as efficiently as the free sugars at concentrations 200-fold lower (34). The reason for this increase in efficiency is believed to be linked to the more efficient transport of highly lipophilic *O*-acetylated ManNAc through the cell membranes as compared to unmodified ManNAc. In our studies, Sf9 cells co-infected with AcKin and AcSAS with 0.2 mM Ac<sub>4</sub>ManNAc were able to synthesize Neu5Ac at levels comparable to cells co-infected with AcEpimKin and AcSAS and supplemented with 0.2 mM GlcNAc or cells co-infected with AcKin and AcSAS with ManNAc feeding levels 50 times higher at 10mM. Thus, Neu5Ac synthesis for AcSAS-infected Sf9 cells is limited at the point of ManNAc transport for almost all concentrations of fed ManNAc since the Neu5Ac levels obtained when using 0.2 mM Ac<sub>4</sub>ManNAc exceeded those obtained for all ManNAc supplementation levels up to 10 mM. In contrast, the limitation in ManNAc kinase activity is only clearly apparent at levels of ManNAc exceeding 20mM. *O*-acetylation is a way of increasing efficiency of transport but it can not be used at

higher concentrations because of its toxicity. A method for improving ManNAc transport efficiency without any toxic side-effects may be beneficial for further enhancing the sialic acid metabolism from fed ManNAc.

Substrate feeding and metabolic engineering may also be used to control the type of sialic acid formed. The alternate sialic acid, KDN, is formed in addition to Neu5Ac when cells are infected with AcSAS (15). While Neu5Ac is derived from ManNAc-6-P, KDN is generated by the same enzyme from Man-6-P (Fig. 1B). Furthermore the levels of KDN synthesized increased when the cells lack the ManNAc-6-P precursor for Neu5Ac synthesis. One way to lower the ManNAc-6-P level is to eliminate ManNAc supplementation to the media and another is to inhibit the intracellular formation of ManNAc-6-P. As Sf9 cells depend on GlcNAc kinase for phosphorylation of ManNAc, the synthesis of ManNAc-6-P can be restricted by feeding GlcNAc to the cells. Since the GlcNAc kinase preferentially acts on GlcNAc, this activity inhibits its ManNAc kinase function. Indeed, the addition of 10 mM GlcNAc was shown to favor KDN synthesis with little or no trace of Neu5Ac synthesis. In the absence of any available ManNAc-6-P in AcSAS-infected cells, the sialic acid 9-phosphate synthase enzyme acts on the available Man-6-P and converts it to KDN. However, the total levels of KDN obtained intracellularly were several orders of magnitude below those of Neu5Ac obtained when the AcSAS-infected cells were supplemented with 10 mM ManNAc. This low amount of cellular KDN synthesis may limit the feasibility of generating significant levels of glycoproteins terminating in KDN.

The current study has demonstrated that metabolic bottlenecks can exist in the production of sialic acids in engineered insect cells at the levels of expressed pathway genes and in the availability of substrates for the production of sialic acids Neu5Ac and KDN. These bottlenecks can be overcome by modifying the sialic acid pathway to include critical enzymes that are missing or at low levels and by altering the substrate or substrate transport to maximize sialic acid production. Previous studies with mammalian cells have revealed limitations in the sialylation pathway that can affect the level of sialylation observed on *N*-glycans (35). Insect cells represent an excellent model to evaluate sialylation bottlenecks since many of the genes are missing or expressed at very low levels in these cells.

The critical steps limiting complete sialylation of insect cell-derived glycoproteins are the generation and transport of the nucleotide sugar, CMP-Neu5Ac, the expression of transferases to yield acceptor substrates terminating in galactose, and the expression of sialyltransferase for transfer of Neu5Ac onto galactosylated acceptors. Previous research has shown that exogenous ManNAc feeding in combination with SAS and CMP-SAS expression will generate the essential nucleotide sugar for sialylation, CMP-Neu5Ac, as well as the alternative sugar, CMP-KDN (15). The expression of GalT in combination with GlcNAc T-II yields acceptor substrates terminating in galactose on both branches (11). Recently, the genes for these two bottlenecks were combined with sialyltransferase expression to generate fully sialylated glycoproteins for insect cells grown in serum-free medium (36). Apparently, insect cells can achieve full sialylation without expression of the transporter that moves the CMP-Neu5Ac into the golgi apparatus. However, these studies were performed in medium supplemented with exogenous ManNAc. The current study shows that the requirement for any medium supplementation can be eliminated completely by expressing the UDP-GlcNAc-2-epimerase/ ManNAc kinase gene in combination with other sialic acid pathway genes. Adding the UDP-GlcNAc-2-epimerase/ ManNAc kinase gene to the others listed above will enable insect cells to become, to our knowledge, the first non-mammalian species to generate fully sialylated glycoproteins completely from intracellular metabolic components. Furthermore, the absence of these genes in insect cells has enabled the elucidation of specific bottlenecks in the sialic acid synthesis pathway. Critical bottlenecks including intracellular ManNAc transport as well as phosphorylation were identified to suggest ways in which production of CMP-Neu5Ac may be enhanced in the future. The elucidation of particular bottlenecks may be applicable to mammalian cell cultures as well since some of these pathway steps may also be limiting in these cells (17). Metabolic engineering efforts like the ones described here will enable the optimization of intracellular pathways in insect cells, mammalian cells, and perhaps other species in order to achieve desirable levels of sialylation and other glycosylation modifications in the future.

#### Acknowledgement

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## Legends

**Figure 1:** Schematic of the sialic acid biosynthetic pathways in mammalian cells. (A) Pathway for synthesis of Neu5Ac and CMP- Neu5Ac in mammalian cells. (B) Steps in the synthesis of KDN and CMP-KDN in mammalian cells.

**Figure 2:** Effect of different ManNAc concentrations on Neu5Ac and KDN levels in Sf9 insect cells. (A) Intracellular Neu5Ac levels for cells infected with AcSAS virus or left uninfected in the presence of varying quantities of ManNAc. The cells were harvested 3 days post infection and Neu5Ac levels were measured by DMB labeling. The right panel includes the Neu5Ac synthesis pathway. The bold arrows represent recombinant activity and dashed arrows represent native activity. An X over the arrow represents a step in the pathway for which the enzyme activity is either negligible or not present at all. (B) Intracellular Neu5Ac levels for cells infected with A35 (negative control virus) or left uninfected in media supplemented with varying quantities of ManNAc. The cells were harvested at 3 days post infection and the intracellular Neu5Ac levels were measured as before. (C) Absolute and relative KDN production levels for Sf9 cells infected with A35 (negative control virus) or AcSAS in medium supplemented with different ManNAc quantities. Left axis and closed symbols refers to intracellular KDN content measured by DMB labeling. Right axis and open symbols refers to the ratio of KDN level to Neu5Ac level. All experiments were performed in duplicate with similar trends.

**Figure 3:** Schematic of UDP-GlcNAc-2-epimerase/ ManNAc kinase bifunctional gene along with mutants. The region of epimerase activity is located at the amino terminus of the protein while the kinase active site is localized to the carboxy terminus of the protein. The AcEpimKin virus includes domains for fully functional epimerase and kinase activities. The AcKin virus contains an active kinase domain with a mutation in the epimerase domain (H45A) to eliminate epimerase activity. The AcEpim virus includes a functional epimerase gene with a mutation in the kinase domain (R420M).

**Figure 4:** Sialic acid production as measured by HPLC detector for Sf9 cells infected with AcSAS and AcEpimKin compared to production following infection with AcSAS alone in media supplemented with 10 mM ManNAc. Also included as a negative control is the activity following infection with AcEpimKin alone. Neu5Ac and KDN levels were measured following DMB derivatization using a fluorescence detector with a reverse phase HPLC setup. Detector responses on chromatograms have been normalized using total protein concentration in lysates for each sample.

**Figure 5:** Effect of various HexNAc (GlcNAc or ManNAc) supplementation levels on Neu5Ac levels in Sf9 cells infected with AcSAS. Cells co-infected with AcEpimKin and AcSAS were supplemented with GlcNAc while cells co-infected with AcKin and AcSAS were supplemented with ManNAc. All experiments were performed in duplicate.

**Figure 6:** A comparison of Neu5Ac levels for Sf9 cells co-infected with AcSAS in the presence and absence of AcKin for various levels of ManNAc supplementation. Cell infected with the virus were harvested 3-days post infection and the lysates were analyzed for Neu5Ac. Experiments were performed in duplicate with similar trends.

**Figure 7 (A)** The modification of the hydroxyl groups of ManNAc by *O*-acetyl groups leads to the generation of tetra-*O*-acetylated versions of ManNAc ( $\text{Ac}_4\text{ManNAc}$ ). (B) Levels of Neu5Ac in Sf9 cells co-infected with AcSAS and AcKin in the presence of  $\text{Ac}_4\text{ManNAc}$  compared to levels with GlcNAc feeding following AcEpimKin and AcSAS co-infection and AcKin + AcSAS co-infections with ManNAc feeding. Infection with AcSAS supplemented with 10 mM ManNAc was also included as a comparison. Experiments in Fig. 7B were performed multiple times with similar results.

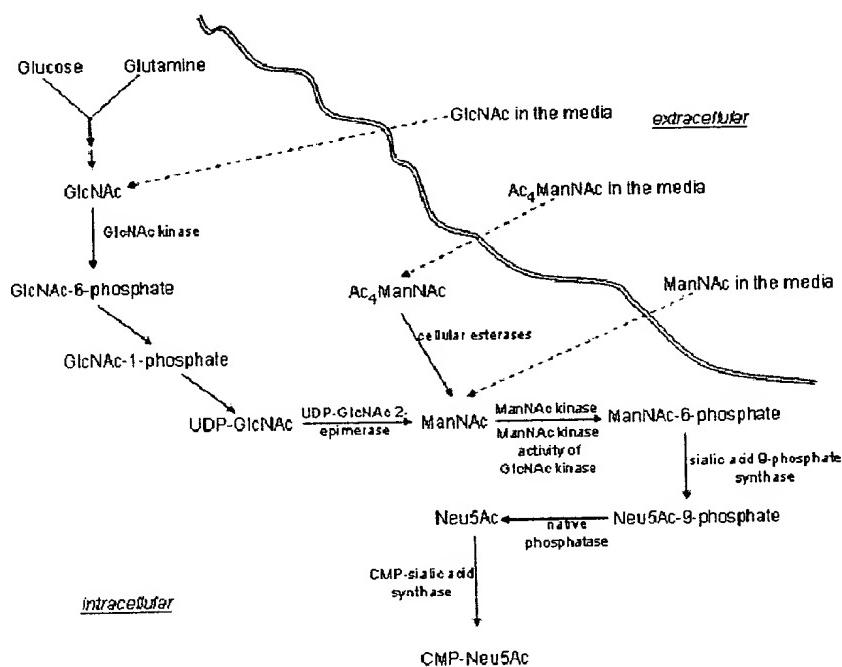
**Figure 8:** Inhibition of Neu5Ac synthesis in Sf9 cells caused by feeding GlcNAc. Neu5Ac and KDN levels were measured in Sf9 cells supplemented 10mM GlcNAc following AcSAS + AcEpim co-infection in the presence or absence of AcKin.

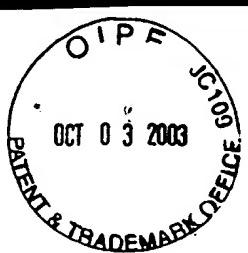
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Figure 1A



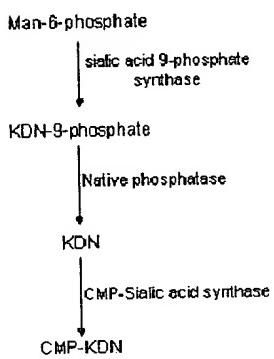


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Figure 1B

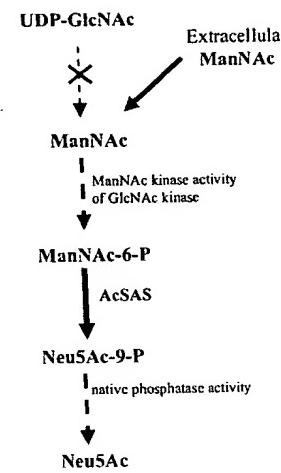
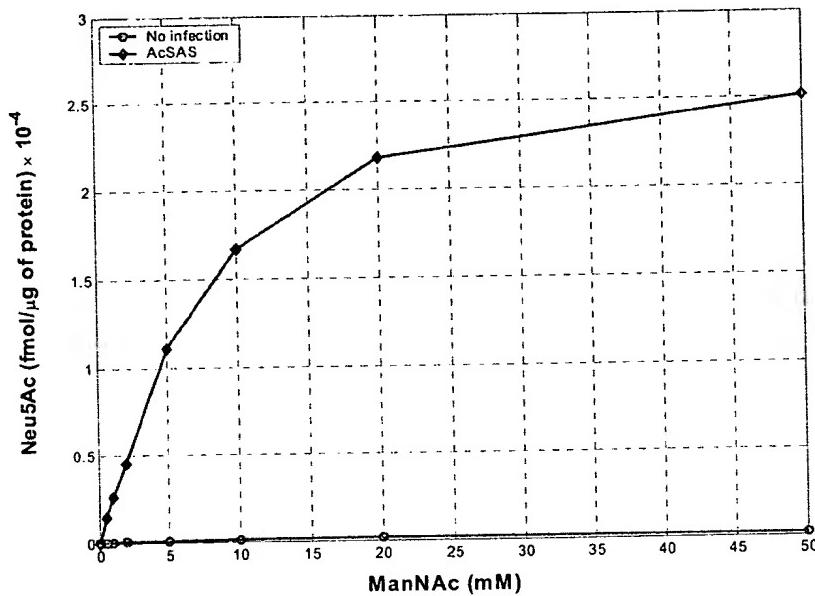




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Figure 2A

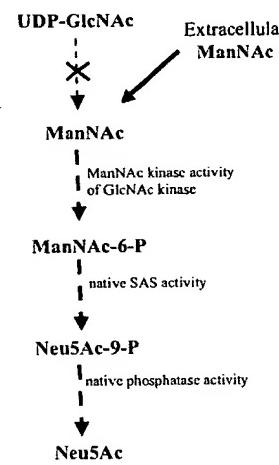
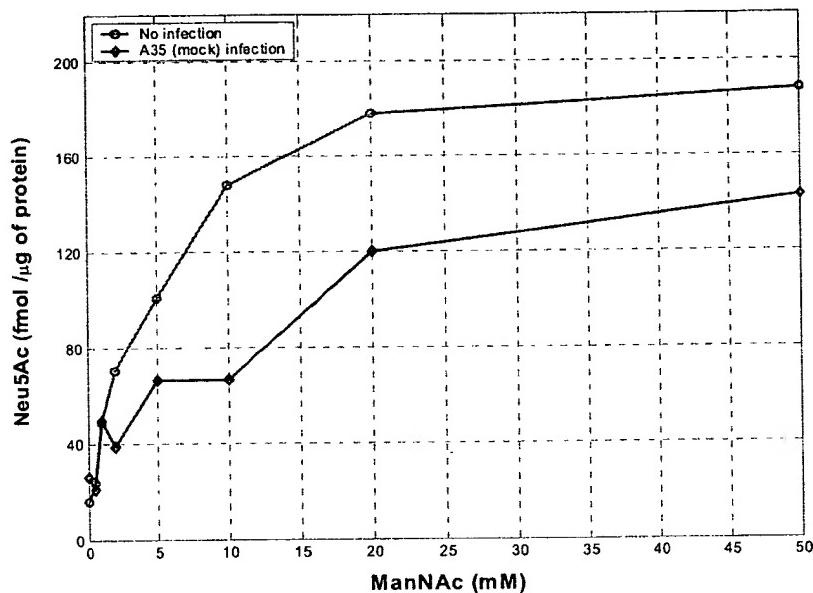


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Figure 2B

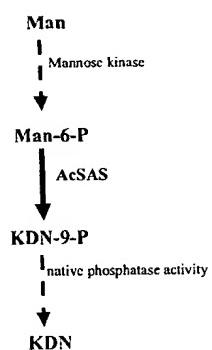
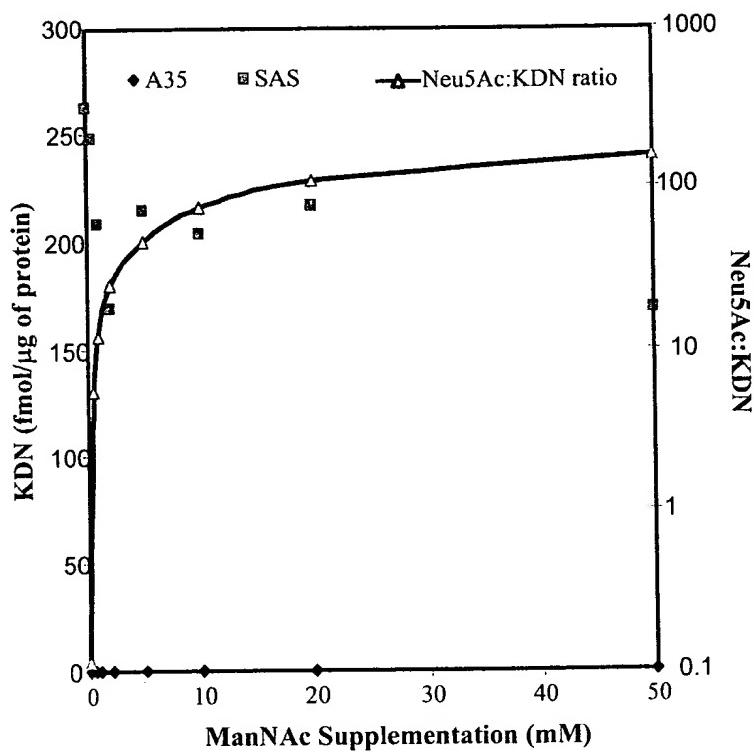


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Figure 2C



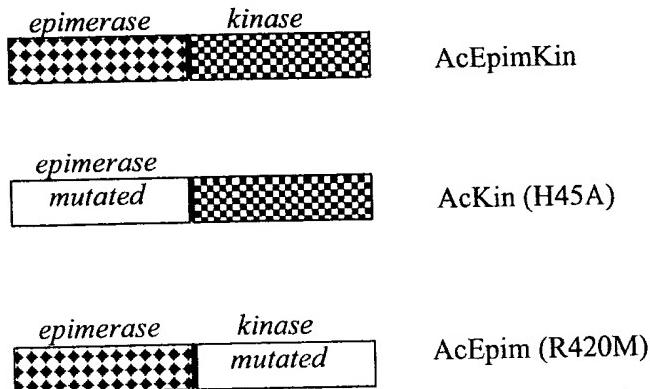


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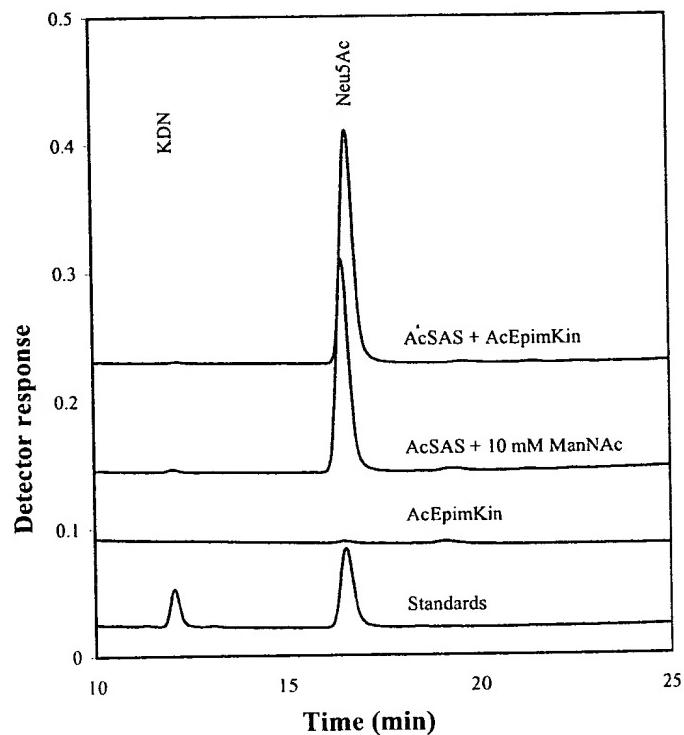
Figure 3



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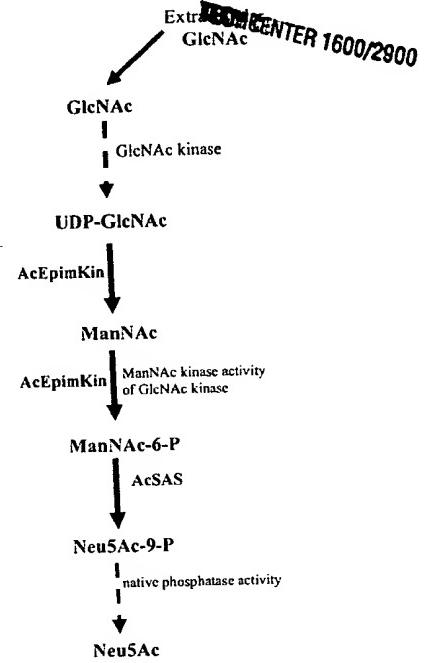
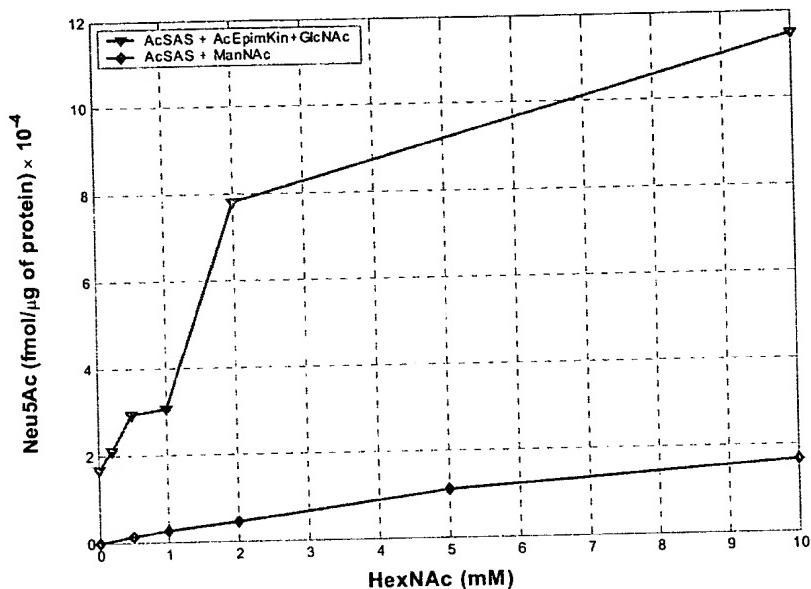
Figure 4



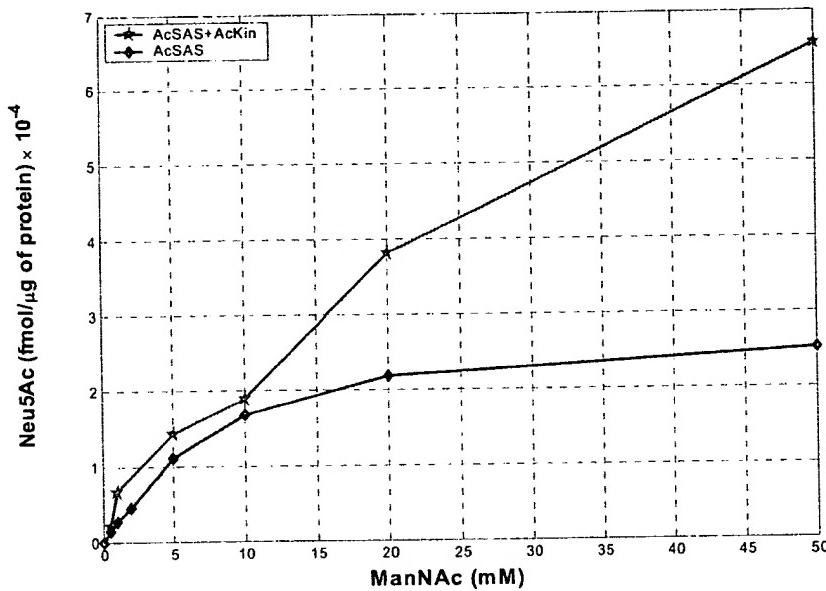
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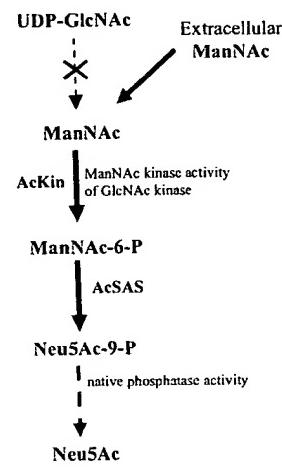
Figure 5



**Figure 6**



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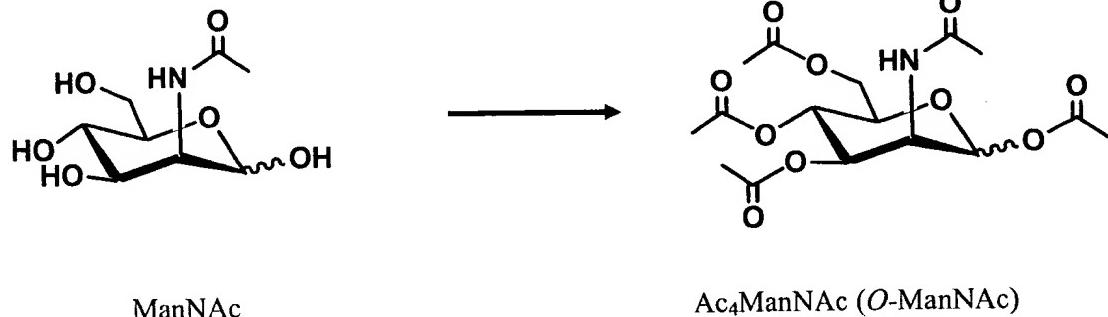


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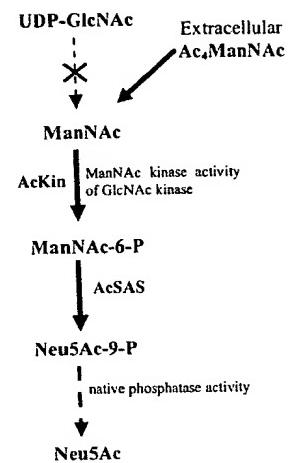
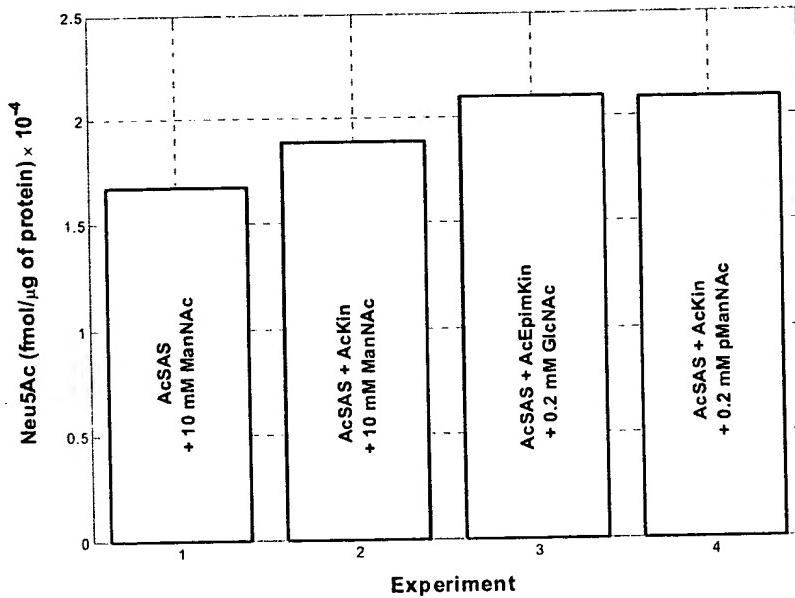
Figure 7A



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Figure 7B



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Figure 8

